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REVIEW

Gene Therapy (1994) 1, 223–232

Strategies to achieve targeted gene delivery via the receptor-mediated endocytosis pathway

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Gene transfer to eukaryotic cells may be accomplished by capitalizing on endogenous cellular pathways of macromolecular transport. In this regard, molecular conjugate vectors have been developed which deliver DNA via the receptor-mediated endocytosis pathway. An attractive feature of this vector system is the potential to achieve targeted gene delivery based upon flexible incorporation of a targeting ligand. In this review we describe steps that have been taken to optimize this vector system. Specific strategies include the incorporation of mechanisms to achieve conjugate escape from the endosome and the derivation of methods to eliminate sources of nonspecificity. These developments have demonstrated the potential to construct a vector system in which multiple independent components may function in a concerted manner to accomplish targeted high efficiency gene delivery. In their present state of development, molecular conjugate vectors may have many potential applications for in vitro use.

Introduction

There are presently two gene transfer strategies to accomplish gene therapy: *ex vivo* and *in vivo*. Despite their efficacy in selected contexts, *ex vivo* strategies are limited to cells that can be manipulated extracorporeally. Thus, the ability to accomplish *in vivo* gene transfer would significantly expand practical applications of various gene therapy strategies. To accomplish *in vivo* gene transfer, a vector must possess certain intrinsic characteristics. It must first be capable of efficient gene delivery. Despite the large number of gene transfer vehicles currently available, very few are able to mediate effective gene delivery *in vivo*. This reflects the fact that in order to accomplish *in vivo* intracellular delivery, the gene transfer vector must first be able to transit various systemic barriers. In addition to *in vivo* efficiency, a systemically administered vector should also possess the capacity to mediate gene expression in a selected cell population. In this regard, a targetable gene transfer vector should only be internalized into a cell type of interest and the delivered gene expressed only in that cell line. An ideal systemic gene transfer vector would also be devoid of viral gene sequences. This strategy would circumvent safety issues intrinsic to viral gene delivery systems which would be of even greater significance in the setting of a systemically delivered vector. A gene transfer vector meeting the above criteria would be in conceptual accord with the proposed 'targetable, injectable vector' [1–4]; a safe, non-toxic and efficient *in vivo* delivery vector, and would dramatically expand the practical applications of gene therapy.

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Many different types of vectors have been developed for gene transfer applications. These vectors include viral vectors, such as recombinant adenoviruses and retroviruses, and non-viral vectors, such as liposomes and calcium phosphate precipitation. In all of these strategies, in order to accomplish effective gene transfer, the delivered gene must cross multiple subcellular barriers. These barriers include: transit through the eukaryotic cell plasma membrane, cytoplasmic to nuclear transport, nuclear entry and functional maintenance within the nucleus. Persistence of gene expression can be achieved either by stable integration of heterologous DNA in the nucleus or by maintenance of an extrachromosomal replicon. Therefore, an effective gene transfer vector must possess specific mechanisms to accomplish each of the aforementioned steps.

Viral vectors

Viruses have evolved specifically to transport their genetic material to target cells using mechanisms both intrinsic to the virion and also expropriated from the target cells [5–7]. In this regard, viral vectors such as recombinant adenoviruses have been developed which capitalize on the efficient internalization pathway of these viruses [8]. Recombinant adenoviral vectors are designed so that the gene of interest is integrated within the viral genome. In this configuration, viral gene delivery also accomplishes delivery of heterologous genes. Thus, the integrity of the viral genome is crucial to the vector function of therapeutic gene delivery. Despite the ability of adenovirus vectors to accomplish gene delivery both *in vitro* and *in vivo* [8–10], recombinant adenoviral vectors are limited by both practical and theoretical constraints. In practice, the amount of therapeutic DNA sequences which may be

packaged is limited to only 6–8 kb [8, 11]. Thus, gene therapy strategies for diseases such as muscular dystrophy may be limited using adenoviral vectors due to the large size of the gene of interest required to correct this disease [12]. In addition, the targetability of recombinant adenoviral vectors is limited by the tropism of the virus. This can have two possible implications. On one hand, ectopic gene delivery may be accomplished to cell types which contain adenovirus receptors; the adenovirus cell surface receptor is endogenous to many cell types and promiscuously expressed [10]. On the other hand, the cell surface receptor may not be found in the cell type of interest as is the case with differentiated muscle cells [12]. In addition to targetability, the issue of vector safety is undefined. Since the viral genome is crucial to vector function, obligatory co-delivery of viral genes introduces potential safety risks. In spite of some of these limitations, recombinant adenoviral vectors are currently being employed in selected human protocols.

Like recombinant adenoviruses, recombinant retroviruses transduce cells via a highly efficient internalization pathway. Recombinant retroviral vectors have been designed to capitalize on the fact that integration of the parental genome is a normal part of the life cycle of the virus [13]. The capacity to integrate exogenous DNA sequences with subsequent long-term gene expression is highly desirable in many therapeutic scenarios. In selected contexts, recombinant retroviruses can effectively transduce certain cell populations. However, recombinant retroviruses, like recombinant adenoviruses, are limited by the amount of exogenous DNA they can package; only 6–7 kb [14]. In addition, they are unable to transduce cells which are terminally differentiated, thus limiting their *in vivo* efficacy [15]. In addition, the obligatory introduction of elements of the parent virus genome during delivery poses potential safety hazards as a consequence of recombination events with endogenous human retroviruses generating replication-competent viruses [14].

Non-viral vectors

As an alternative to viral systems, non-viral gene transfer vectors have been developed. Non-viral gene transfer vectors utilize distinct mechanisms to accomplish gene delivery. For example, liposomes deliver DNA by membrane fusion [16], $CaPO_4$ precipitation by non-specific endocytosis [17] and direct DNA injection by physical disruption of the membrane [18]. There are two major limitations intrinsic in the design of these non-viral gene transfer vectors. The first is that gene delivery employing these methods results in membrane perturbation. Disruption of cellular membranes is intrinsically cytotoxic, thus making these vectors unlikely candidates for *in vivo* gene therapy. Second, these non-viral vectors are designed to deliver exogenous DNA to the cytosol. Without a mechanism to transport the gene to the nucleus for subsequent gene expression, efficiency is limited. In addition, as these methods transduce cells indiscriminately, it may not be possible to use these non-viral vectors to target specific cell types.

Molecular conjugate vectors

To circumvent these limitations, methods have been developed to deliver DNA via the receptor-mediated endocytosis pathway [19–24]. These vectors, called molecular conjugate vectors, capitalize on the internalization mechanism intrinsic to specific macromolecules; receptor-mediated endocytosis. The basic design of molecular conjugates is to attach plasmid DNA to a macromolecule ligand which can be internalized by the cell type of interest. To accomplish this, a molecular conjugate vector possesses two distinct functional domains: a DNA binding domain which is composed of a polycation such as polylysine and a ligand domain which binds to a particular cell surface receptor. The polylysine domain is chemically linked to the ligand [19, 21]. The polycation not only binds DNA through electrostatic forces but condenses the DNA allowing it to mimic the compact structure of macromolecules [25]. This facilitates the entry of DNA into the cell vesicle system.

Conjugate vectors which internalize by a normal cellular pathway were first developed by Wu *et al.* to achieve gene transfer in hepatocytes [19, 20]. In this strategy, gene transfer to hepatocytes was achieved using the internalization mechanism for clearance of asialoglycoproteins, which are internalized by hepatocytes. Other groups have also achieved gene transfer by receptor-mediated strategies. Birnstiel *et al.* have accomplished gene transfer via receptor-mediated endocytosis using transferrin–polylysine molecular conjugate vectors as shown in Figure 1 [21–23]. In addition, Davis has achieved gene transfer via the immunoglobulin A (IgA) transcytosis pathway as a method to target respiratory epithelial cells [24].

Molecular conjugate vectors possess potential advantages for gene therapy applications. First, since vector entry is via a normal physiological pathway, transduction is non-toxic to cells and the vector can be administered repeatedly. Second, as conjugates are devoid of any viral elements, this eliminates the possibility of deleterious recombinational events. In addition, due to the plasticity of their design, it is theoretically possible to target any cell type by the choice of the ligand domain. Thus, this vector system uniquely allows for the capacity of cell-type specific targeting.

Despite their theoretical advantages over other gene transfer vectors, gene transfer efficiency in several *in vitro* systems has been idiosyncratic. For example, delivery of molecular conjugates to cells which appeared to have the appropriate cell surface receptors frequently resulted in insignificant gene expression [22, 26]. In those instances, fluorescent imaging of cells which were transfected with molecular conjugates and yet showed absent heterologous gene expression indicated that the conjugates were indeed internalized but appeared to be trapped within endosomal vesicles [23]. To increase the amount of DNA that escapes lysosomal degradation, different lysosomatropic agents have been used to augment expression of transfected DNA [23]. These findings are consistent with the fact that, although molecular conjugates possess a specific and efficient internalization

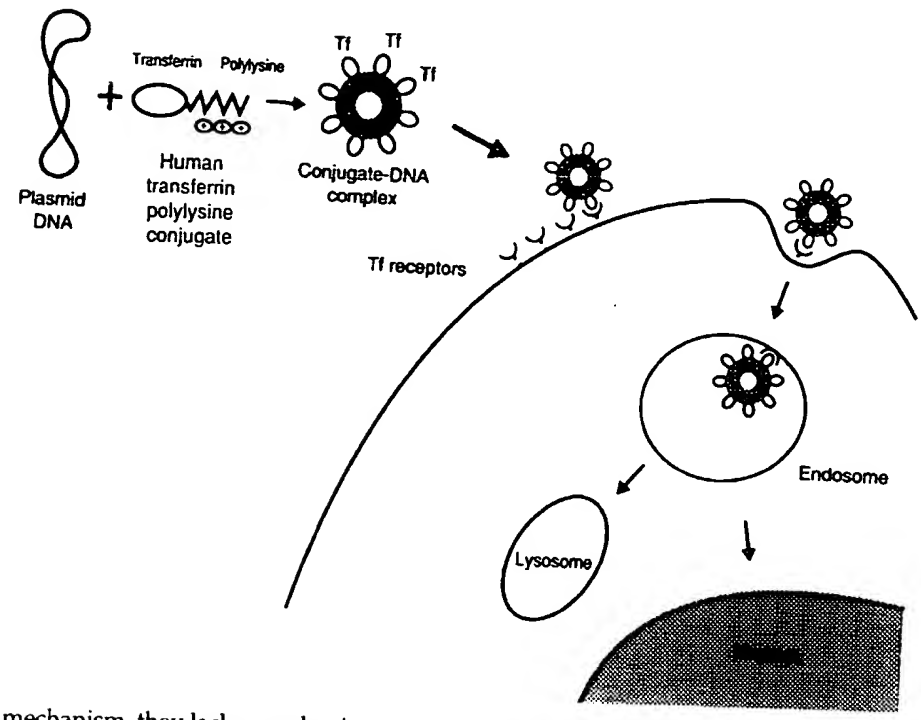


Figure 1 Gene transfer via the receptor-mediated endocytosis pathway. A bifunctional molecular conjugate is employed to bind DNA and transport it via cellular macromolecular transport mechanisms. The conjugate consists of a DNA-binding domain, comprised of a cationic polylysine moiety, which is covalently linked to a ligand for a cell surface receptor, in this case transferrin. Plasmid DNA bound to the polylysine moiety of the conjugate undergoes marked condensation to yield an 80–100 nm toroid with surface-localized transferrin molecules. When the transferrin ligand domain is bound by its corresponding cell surface receptor, the conjugate is internalized by the receptor-mediated endocytosis pathway, co-transporting bound DNA. Escape from the cell vesicle system is achieved by a fraction of the internalized conjugate–DNA complex to achieve nuclear localization where heterologous gene expression is achieved. Reproduced from 'Progress in Medical Virology', Vol 40 pp. 1–18 (1993) with permission of S. Karger.

mechanism, they lack a mechanism to accomplish escape from lysosomal degradation after cellular internalization which limits gene transfer efficiency.

Adenoviruses facilitate molecular conjugate delivery

To overcome the limitation of endosome entrapment, a strategy was explored with the goal of augmenting gene transfer by the receptor-mediated endocytosis pathway. In this regard, adenoviruses enter cells by a receptor-mediated endocytosis pathway [27, 28]. The entry pathway of adenoviruses is, in fact, quite similar to the initial entry events of molecular conjugates. The adenovirus fiber protein binds to an uncharacterized cell surface receptor [27]. The virion is then engulfed into clathrin-coated pits and internalized into an endosome. Unlike molecular conjugates, adenoviruses are not trapped within these vesicles. Acidification of endosomes containing adenovirus particles results in endosome disruption [29], allowing virions to proceed to the nucleus to complete their life cycle.

It is noteworthy that the process of vesicle disruption is a function of viral capsid proteins and independent of viral gene expression [28]. It has been hypothesized that endosome acidification during adenovirus internalization elicits conformational changes in capsid proteins in a manner that allows the capsid proteins to interact with and disrupt the vesicle membrane. The exact mechanism of endosome disruption has not been completely delineated. However, monoclonal antibodies against the penton protein have been shown to selectively block endosome lysis [30]. Thus, the penton base and/or peripentonal capsid components appear to be the crucial capsid proteins involved in endosome disruption. Inferential evidence also suggests that functional cooperativity among other capsid proteins may be crucial in this process.

It has been shown that adenovirus can facilitate the cellular entry of macromolecules [31]. Furthermore, it has been established that receptor-bound ligands can be co-internalized with adenovirus [32]. In this regard, Pastan *et al.* have shown that adenovirus can facilitate chimeric toxin-conjugate entry via the receptor-mediated endocytosis pathway [32]. In this study, it was shown that this adenoviral facilitation was based upon the endosome disruption capacity of adenovirus. Thus, adenovirus can enhance cytoplasmic delivery of macromolecules subsequent to their endosome co-localization with adenovirus and, furthermore, this capacity of the adenovirus is based upon its ability to disrupt cellular endosomes during cell entry.

These studies suggested the possibility that adenovirus could also facilitate the entry of molecular conjugate vectors by a similar mechanism. To test the hypothesis that adenoviruses could mediate molecular conjugate escape from lysosomal degradation (Figure 2), molecular conjugates containing a luciferase reporter gene were co-delivered to HeLa cells with a replication defective adenovirus [33]. The replication defective adenovirus was used in order to separate effects mediated by viral entry and viral gene expression. It was shown that increasing reporter gene expression corresponded to increasing concentrations of adenovirus. This adenoviral facilitation achieved levels 2000-fold greater than levels achieved with transferrin–polylysine conjugates alone. As the replication-incompetent virus used in these studies is incapable of intrinsic gene expression, the observed augmentation in gene expression during adenovirus facilitation of conjugate-mediated gene transfer exclusively reflects adenovirus entry features. It is important to note that the number of virion particles required to achieve these levels of augmented gene expression corresponded to

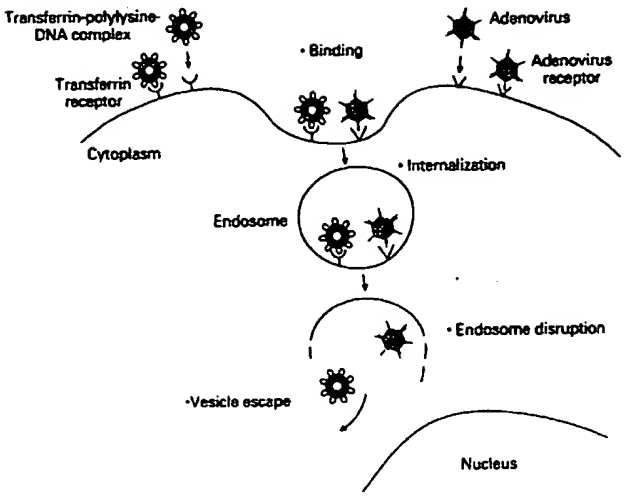


Figure 2 Mechanism of adenoviral facilitation of molecular conjugate-mediated gene transfer. After binding to their respective cell surface receptors, co-internalization of the transferrin–polylysine conjugate and the adenovirus is within the same endocytotic vesicle. Adenovirus-mediated endosome disruption allows vesicle escape for both the virion and the conjugate–DNA complex. Reproduced from 'Progress in Medical Virology', Vol 40 pp. 1–18 (1993) with permission of S. Karger.

the number of adenovirus receptors on the target cell [34]. A saturable dose-dependent effect was observed, as would be expected in a receptor-limiting context. Similar results were noted for a number of target cells, some of which included cell types otherwise refractory to transferrin–polylysine-mediated gene transfer.

Next, the basis of the virus' ability to significantly enhance gene transfer mediated by molecular conjugates was explored. The intention was to be able to differentiate viral entry features which allowed the observed facilitation of conjugate entry. In this regard, heat treatment of adenovirus particles ablated their ability to mediate cell vesicle disruption without impairing the ability to bind to target cells [29]. In these experiments, transferrin–polylysine complexes were co-delivered with heat-inactivated virions. Results indicated that heat treatment completely abrogated the ability of the virus to augment gene transfer. This finding emphasizes the fact that the molecular conjugate's principal limitation to achieving effective gene transfer to target cells is the conjugate's lack of an endosome escape mechanism, and that it is specifically the adenovirus' ability to disrupt endosomes which is responsible for its capacity to augment conjugate-mediated gene transfer.

Adenoviruses linked to molecular conjugate vectors

Since molecular conjugate-mediated gene transfer could be significantly augmented by the addition of free adenovirus particles, it was logical to think that incorporation of adenoviruses into the functional design of the vector would likewise facilitate conjugate-mediated gene transfer through addition of the functional capacity of endosome disruption. The strategy to accomplish this is illustrated in Figure 3. A major concern in trying to link adenoviruses to molecular conjugates was the preservation of both the binding and endosome disruption capabilities of the virus. Since fiber and penton proteins are believed to be primarily responsible for binding and internalization, respectively, and hexon protein is thought to be a 'scaffolding' protein [35], the conjugates were linked through the hexon protein. Linkage was accomplished via an antibody bridge between the molecular conjugate and adenovirus. This was accomplished by conjugating a monoclonal antibody against a foreign epitope on the adenovirus hexon protein to the polylysine–DNA complex (Figure 4). For this purpose, a chimeric adenovirus containing a foreign epitope in the surface region of its hexon protein was constructed [36]. The design of this complex ensured a non-neutralizing linkage between the chimeric virus and molecular conjugates.

The capacity of adenovirus–polylysine–DNA complexes to accomplish gene transfer to target cells was evaluated. In these experiments, different combinations of conjugate components were assayed for their ability to mediate gene transfer. It is important to note that all conjugate components were required to achieve effective gene transfer. Thus, it was in fact the physical linkage of DNA to virus that was the crucial factor allowing high efficiency gene delivery. In these experiments, heat-inactivated adenovirus was unable to mediate high efficiency gene transfer in the linked configuration, also indicating that the ability to mediate endosome disruption is the property of the adenovirus which is responsible for facilitating conjugate entry. These experiments demonstrated that it was possible to design a molecular conjugate vector which contained an integrated endosome lysis domain. Furthermore, this added function greatly augmented the gene transfer capacity of the complexes.

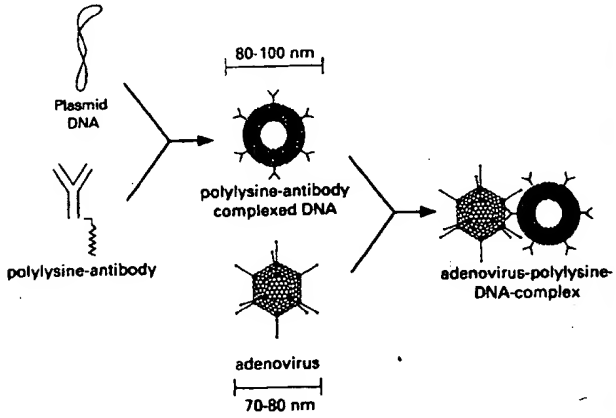


Figure 3 Schematic of approach to derive adenovirus–polylysine–DNA complexes containing heterologous DNA attached to exterior of adenovirus capsid. To accomplish linkage of an adenoviral cognate domain and a polycationic DNA-binding domain, the chimeric adenovirus P202-Ad5 containing a heterologous epitope in the exterior domain of its hexon protein was employed in conjunction with a monoclonal antibody specific for this epitope. Control experiments demonstrated that attachment of the monoclonal antibody was non-neutralizing for adenovirus P202-Ad5. The monoclonal antibody was rendered competent to carry foreign DNA sequences by attaching a polylysine moiety. Interaction of the polylysine–antibody complexed DNA with adenovirus P202-Ad5 occurs via the specificity of the conjugated antibody. Reproduced from 'Progress in Medical Virology', Vol 40 pp. 1–18 (1993) with permission of S. Karger.

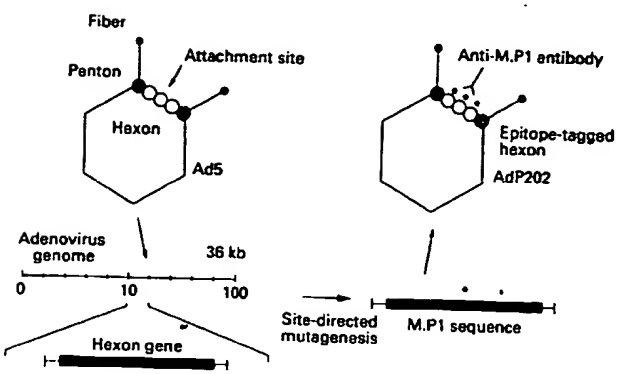


Figure 4 Construction of a chimeric adenovirus containing a heterologous epitope in the surface region of the hexon capsid protein. Since the adenoviral capsid proteins fiber and penton are important mediators of the adenoviral entry mechanism, attachment of capsid-bound DNA was targeted to the hexon protein. A specific attachment site for an immunologic linkage was created by introducing a heterologous epitope into the surface region of the hexon protein by site-directed mutagenesis of the corresponding region of the adenoviral hexon gene. The introduced foreign epitope is a portion of Mycoplasma pneumoniae P1 (M.P1) protein. Reproduced from 'Progress in Medical Virology', Vol 40 pp. 1–18 (1993) with permission of S. Karger.

The delivery strategy of the adenovirus–polylysine–DNA complex is in some way analogous to recombinant viral vectors. However, aspects of the design are novel, differing from recombinant viral vectors in that the heterologous DNA sequence is linked to the outside of the adenovirus. This design confers certain practical advantages. First, there is less constraint in the size of DNA to be delivered as this capacity is not limited by the packaging constraints of the virus. In addition, since the gene of interest binds non-covalently to polylysine in a sequence-independent manner [23], it is possible to carry DNA of any design. Another difference is that recombinant viral vectors are highly dependent on the parent viral genome for all functions related to the delivery of heterologous DNA to the nucleus of target cells. To illustrate the fact that this is not the case in the adenovirus–polylysine–DNA complex, a replication-defective adenovirus was used in the linked complex. No significant decrease in gene transfer efficiency was noted. This again emphasizes the fact that it is the adenovirus capsid proteins that mediate endosome disruption and this event is independent of viral genome function [37].

Significantly, it was observed that adenovirus-linked molecular conjugates were able to mediate levels of gene expression which were two orders of magnitude greater than gene transfer achieved by free adenovirus facilitation of molecular conjugates [38]. Whereas one would expect a facilitation in gene transfer based upon the endosome disruption function of the adenovirus, it was further noted that molecular conjugates with linked adenovirus appeared to be even more facilitated than conjugates co-delivered with free adenovirus. The basis for this may be deduced from considerations of the methods of facilitation in these two instances. In the linked configuration, the adenovirus possesses the capacity to transport the bound DNA to the nuclear pore through its intrinsic nuclear localization capacity. Alternatively, in the free adenovirus configuration, the adenovirus functions as an endosome lysis agent allowing complex DNA to enter the cytosol. In this instance, no facilitation beyond cytoplasmic delivery is provided. Thus, in the linked configuration, it is likely that adenovirus not only mediates vesicle disruption but effectively transports the DNA to the nucleus due to the presence of a nuclear localization function (Figure 5) [27, 28].

Other viruses linked to molecular conjugate vectors

Since adenovirus was able to efficiently augment gene transfer mediated by molecular conjugates, other viral

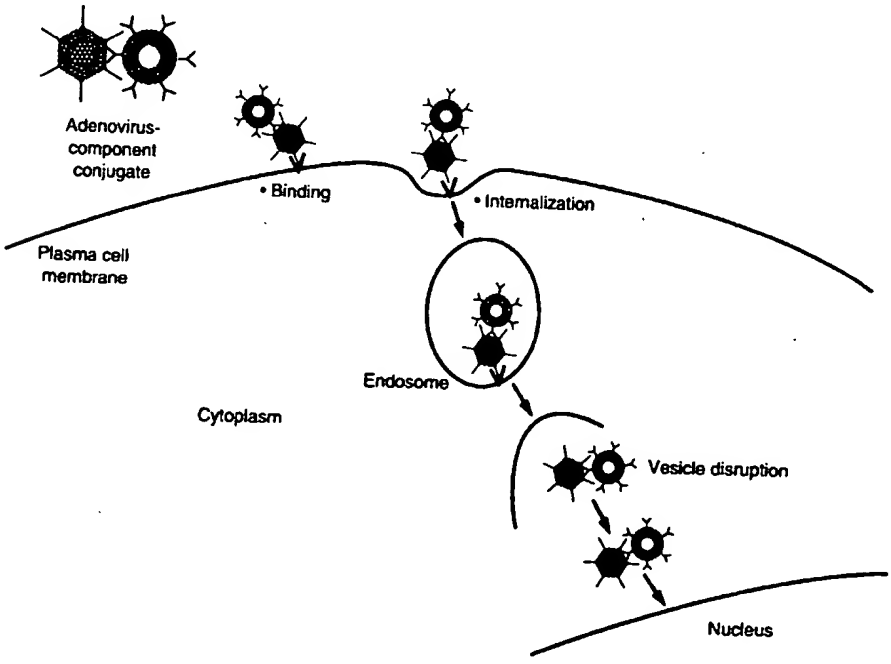


Figure 5 Entry pathway of adenovirus-component molecular conjugate vector. After binding to target cells, the complex is internalized via the receptor-mediated endocytosis pathway. Escape from the endosome is accomplished by adenoviral-mediated disruption of the cell vesicle. This allows the complex to ingress into the cytosol, where it may access the nuclear pore based on adenoviral nuclear localization signals. Reproduced from 'Progress in Medical Virology', Vol 40 pp. 1–18 (1993) with permission of S. Karger.

agents were studied for their ability to mediate similar results. In this regard, the enveloped influenza virus, which also enters cells by a receptor-mediated endocytosis mechanism [5], was linked to the molecular conjugate complex. Inclusion of influenza as the viral component of the conjugate did not result in increased gene transfer efficiency compared with adenovirus complexes. This is presumably due to the differences between the entry mechanisms employed by the different viruses. For influenza, viral gene delivery is carried out through a membrane fusion event between the viral envelope and the cell endosome membrane [5]. It is likely that the complexed heterologous DNA, in this instance, remains trapped within the cell vesicle. Thus, it is not sufficient for the entry mechanism of the viral component to be receptor-mediated endocytosis. A vesicle disruption event seems to be required for enhanced gene transfer efficiency.

Ternary complexes

The viral component in the adenovirus–polylysine–DNA conjugates was added for the purpose of providing an escape from lysosomal degradation. However, the adenovirus may also act as the exclusive ligand domain of the molecular conjugate in this configuration. To validate this concept, different cell types which either contained a large population of adenovirus receptors or were relatively refractory to adenovirus binding were targeted. As anticipated, the tropism of the virus dictated the specificity of the adenovirus–polylysine–DNA conjugates [36]. Cells which contain a high number of adenovirus receptors, HeLa and KB cells, are highly susceptible to gene transfer using these complexes. In contrast, HBE1 and MRC-5 cells, which contain a relatively low number of adenovirus receptors, are much less susceptible to gene transfer by this vector system. Thus, in the adenovirus–polylysine–DNA configuration, the adenovirus component dictated the tropism of the conjugate.

To overcome this potential limitation, it was determined whether the viral component could be used as an endosome lysis agent in conjunction with an alternate ligand domain and, thus, expand the tropism of the vector system. To accomplish this, 'combination' or 'ternary complexes' were derived (Figure 6). These complexes are formed by the sequential addition of adenovirus, antibody–polylysine and DNA, followed by the second ligand, transferrin–polylysine. To determine the gene transfer efficiency of these ternary complexes, both transferrin–polylysine–DNA, and adenovirus–polylysine–DNA complexes, in addition to ternary complexes, were delivered to HeLa cells. This cell line possesses high levels of both adenovirus and transferrin receptors. A marked increase in gene transfer efficiency was observed with the ternary complexes compared with the adenovirus–polylysine–DNA complexes. This augmentation was much greater than any additive effect of adenovirus–polylysine and transferrin–polylysine would predict. This result could be explained by efficient targeting of these complexes through both the adenovirus and transferrin receptors with the adenovirus functioning

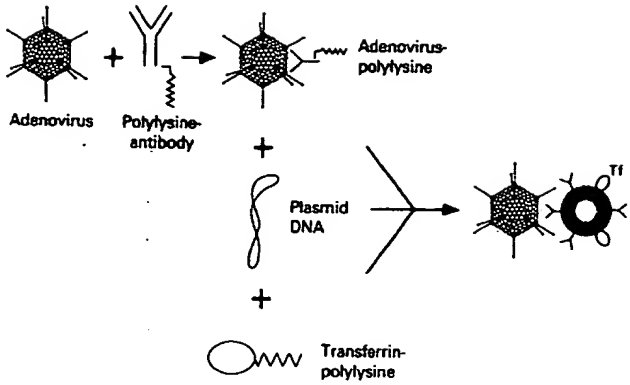


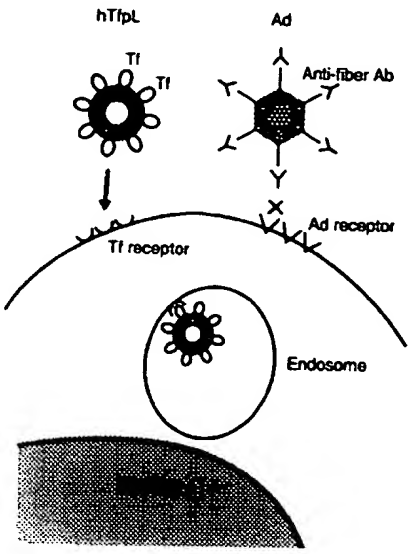
Figure 6 Strategy for the employment of combination conjugates containing adenovirus and transferrin. Complexes were derived that contain transferrin as the ligand domain and adenovirus as an endosome lysis domain. These combination complexes possess the potential to enter cells via the transferrin or adenovirus pathway. In the former instance, after entry via the transferrin pathway, the adenovirus would function exclusively in the capacity of an endosome lysis agent. Such conjugates thus possess both specific internalization and endosome escape mechanisms. Reproduced from 'Progress in Medical Virology', Vol 40 pp. 1–18 (1993) with permission of S. Karger.

as an endosome lysis agent by either pathway. Thus, it is possible to derive complexes which contain distinct endosome disruption and ligand domains which function in a concerted manner to accomplish gene delivery.

To determine whether adenovirus–transferrin–polylysine conjugates could internalize solely by the transferrin pathway with adenovirus still acting as an endosome lysis agent, these complexes were delivered to HBE1 cells which have previously been shown to contain few adenovirus receptors. When HBE1 cells were transduced with these combination complexes, levels of gene expression were comparable to those observed in HeLa cells [36]. In this instance, it was shown that adenovirus could still mediate vesicle disruption even when it is internalized by an alternate pathway. This result demonstrates the utility of providing a second ligand domain to augment gene transfer to cells which may lack adenovirus receptors.

Ablation of adenoviral binding

Combination complexes contain three linked, functionally independent domains which together mediate high efficiency gene transfer in target cells. These domains include: a DNA binding domain, a ligand domain and a viral component which was added to provide utility as an endosome lysis agent. The fact that these complexes provide two distinct internalization mechanisms, however, undermines the potential targeting specificity of molecular conjugate vectors. In order to exploit the endosome lysis ability of adenovirus selectively, monoclonal antibodies (mAbs) directed against adenovirus fiber protein were generated [39]. These antibodies were able to effectively neutralize adenovirus binding and internalization. The effect of neutralizing mAb on the facilitation of molecular conjugate-mediated gene transfer by free adenovirus was then evaluated (Figure 7). It was shown that not only did the anti-fiber antibody ablate adenovirus binding, but it also obliterated its ability to facilitate



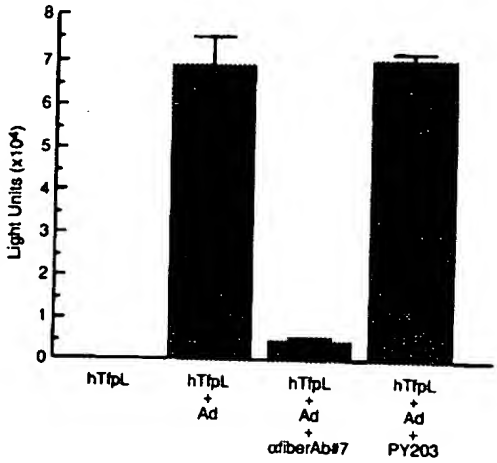


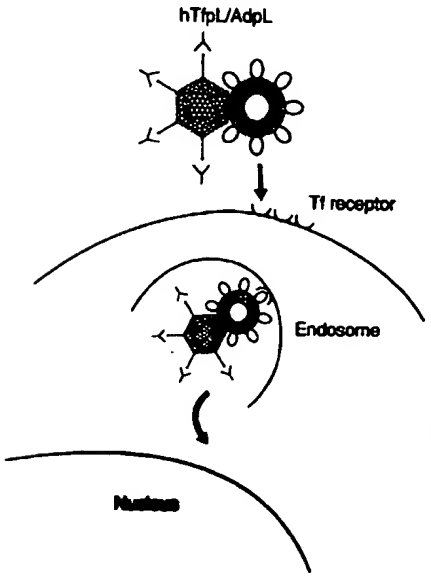
Figure 7 Effect of anti-fiber antibody on the ability of free adenovirus to facilitate molecular conjugate-mediated gene transfer. Cells were treated with human transferrin–polylysine–DNA complexes plus free adenovirus dl312. Adenovirus was used to facilitate gene transfer through its ability to disrupt cellular endosomes, thus allowing conjugate– DNA complex ingress into the target cell cytosol. The adenovirus had been pre-treated with either an anti-fiber antibody (αfiberAb #7) or an irrelevant antibody (PY203). Cell lysates were evaluated for reporter gene expression. Reproduced from 'Gene Therapeutics: Methods and Applications of Direct Gene Transfer' pp. 99–117 (1994) with permission of Birkhäuser.

gene transfer using molecular conjugates. This confirms that adenoviral entry is a prerequisite for viral facilitation of molecular conjugate-mediated gene transfer.

Since the anti-fiber antibody ablated the ability of free virus to facilitate gene transfer using conjugates, it was next determined if ablation of adenovirus binding in the linked configuration would nonetheless allow retention of the endosome disruption capacity of the virus. Although one might expect a decrease in gene transfer based on the loss of one entry pathway, the fact that transferrin receptors are found in excess in HeLa cells would be predicted to make this difference insignificant. In this analysis, the use of antibody-coated, binding-incompetent adenovirus did not decrease the overall levels of gene expression observed (Figure 8). This result indicated that, despite entry via an alternate internalization pathway, fiber binding was not required for adenovirus to mediate endosomal vesicle disruption. This is consistent with the concept that adenoviral binding and vesicle disruption are not functionally linked. Thus, it is possible to construct a multi-functional molecular conjugate that is able to mediate high efficiency gene transfer without having the adenovirus act as a competing ligand. It is thus feasible to exploit the endosome lysis capacity of adenovirus without undermining conjugate specificity.

Other non-specific binding

Ablation of adenoviral binding enhances the overall specificity of a molecular conjugate for a target cell. There are, however, other possible sources of non-specific binding which may arise from the conjugate's design. For instance, it has been shown that polylysine binds non-specifically to certain cell lines [26]. Co-delivery of non-ligand–polylysine–DNA complexes and free adenovirus to cells may result in levels of gene transfer comparable to those observed with ligand–polylysine complexes in these selected instances [26, 33]. Since this non-specific binding is a result of



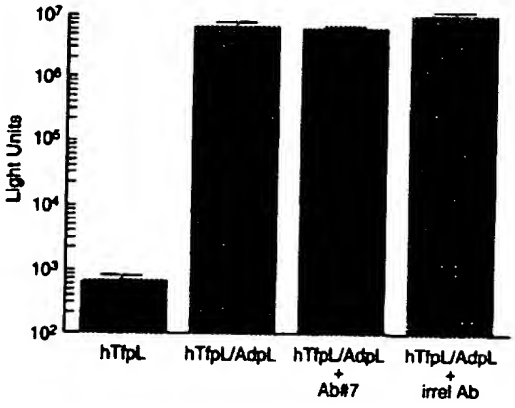


Figure 8 Effect of anti-fiber antibody on the ability of combination complexes to mediate gene transfer. Combination complexes were prepared containing human transferrin and adenovirus (hTfpL/AdpL). The incorporated adenovirus had been pre-treated with either an anti-fiber antibody (αfiberAb #7) or an irrelevant antibody (PY203). Cell lysates were evaluated for reporter gene expression. Reproduced from 'Gene Therapeutics: Methods and Applications of Direct Gene Transfer' pp. 99–117 (1994) with permission of Birkhäuser.

electrostatic interactions between polylysine and cell membranes, it was hypothesized that one could neutralize the charge differential and thus eliminate the basis of this binding. To address this, yeast tRNA, a negatively charged polynucleotide, was used to treat polylysine condensed DNA. The tRNA-treated complexes and free adenovirus were co-delivered to target cells and analyzed for their ability to achieve gene transfer. It was shown that non-specific polylysine–DNA uptake in HeLa cells could be significantly reduced by the tRNA (Figure 9). To show that this treatment did not also block specific uptake by virtue of the ligand, the experiment was repeated using transferrin–polylysine–DNA complexes. This study indicated that tRNA did not interfere with specific ligand uptake. Thus, non-specific polylysine binding to cell surface components can be reduced using a polyanion such as yeast tRNA, without compromising the specific receptor-mediated endocytosis uptake of ligand–polylysine complexes. This modification increases the potential for cell-specific gene transfer using molecular conjugate vectors.

Application of adenovirus-linked molecular conjugate vectors

For certain applications, adenovirus-linked molecular conjugate vectors are ideal in their present form. For example, they have been found to be highly efficient transfection reagents for many *in vitro* applications [21–23, 40]. In addition to mediating high efficiency gene transfer in transformed human cells, they are also able to mediate high efficiency gene transfer in primary cell cultures of various tissue types [41–44]. This is of significance in *ex vivo* transduction of cells which are to be retransplanted after transfection with the gene of interest. Another important property of molecular conjugate design is its ability to deliver large DNA constructs, which is a great limitation in recombinant viral systems. This allows the use of specific promoter elements to enhance overall gene expression. In addition to delivering large genes efficiently, it is also possible to deliver multiple DNA constructs simultaneously.

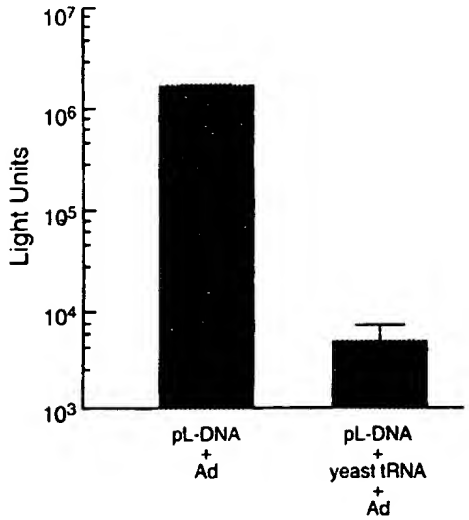


Figure 9 Effect of yeast tRNA on non-specific binding of polylysine-DNA complexes to target cells. HeLa cells were treated with polylysine–DNA complexes (pL–DNA) or tRNA-coated polylysine–DNA complexes (pL–DNA + tRNA) plus free adenovirus dl312 (Ad). After binding at 4°C, plates were washed to remove unbound components and cell lysates evaluated for expression of the luciferase reporter after 24 h. Reproduced from 'Natural Immunity: Potential of Gene Therapy in Cancer' Vol. 13, pp. 141–164 (1994) with permission of S. Karger.

Despite their efficacy *in vitro*, use of molecular conjugates *in vivo* has been idiosyncratic. Gao *et al.* have shown that molecular conjugate vectors are able to mediate gene transfer in the airway epithelium of cotton rats [45]. The observed gene transfer was not, however, as efficient as would be expected from the observed *in vitro* efficacy. This has been shown to be due to the instability of molecular conjugates *in vivo*. It has been shown that the polylysine component of the conjugates is a target of humoral factors after *in vivo* delivery (E. Wagner, personal communication). As the basis of conjugate instability is understood, several steps may potentially be taken to address this problem. As the polylysine component is the primary locus of conjugate instability *in vivo*, it is logical to propose that replacement of the polylysine DNA binding moiety with some other DNA binding component may obviate this limitation. An alternative strategy would be to directly link the DNA to the conjugate via chemical techniques using cross-linking reagents or with a high affinity biotin–streptavidin linkage. In addition, a conceptually distinct strategy being developed seeks to mask the polycation component of conjugates using 'stealthing' procedures. These are several examples of steps that may be taken to improve the *in vivo* efficiency of molecular conjugate vectors by mitigating the known basis of conjugate instability.

In addition to these considerations, other factors could potentially limit the utility of molecular conjugate vectors for strategies requiring *in vivo* delivery by the systemic route. The initial applications of direct *in vivo* targeting of gene transfer utilizing molecular conjugates have involved selective delivery to the liver [46–48]. In this instance, the vascular endothelium of the liver possesses fenestrations of a size which would be predicted to allow conjugate access to the liver parenchyma. For other end-organ targets, however, this may not be the case. The issue of vector immunogenicity could also limit the use of molecular conjugates in applications which require repetitive gene delivery. In this regard, it has recently been shown that systemic delivery of recombinant adenoviruses leads to a prominent host immune response which limits the subsequent capacity for direct *in vivo* delivery employing this strategy [49]. For adenovirus-component molecular conjugate vectors, this phenomenon would probably be of similar significance.

In their present form, molecular conjugate vectors are able to mediate high efficiency gene transfer *in vitro* via the receptor-mediated endocytosis pathway and thus possess the capacity for targeted delivery. This is largely due to the plasticity of the conjugate design whereby incorporated ligands determine the vector tropism. It has been shown that viral endosome disruption functions

incorporated into the conjugate design dramatically enhance gene transfer efficiency, and that these functions may be exploited selectively in a manner whereby the viral tropism will not undermine conjugate specificity. In addition, since the endosome lysis ability of the adenovirus is not a function of viral gene expression, it is possible to selectively exploit this function without introducing viable viral gene elements and thus take steps to inactivate the viral genome. This strategy seeks to capitalize on the minimal functional elements of the virus entry pathway which are useful for vector utility. Although not ideal in its present form, the vector design is consistent with the concept of the proposed targetable, injectable gene transfer vector. It is flexible in its tropism and is largely comprised of specifically derived functional components. The next logical step in vector development is the incorporation of an integration mechanism into the design of the vector. This would enable the delivered DNA to be stably maintained either in the host genome or extrachromosomally leading to long-term gene expression. As it has been demonstrated that distinct functional domains may operate in an independent manner in the context of molecular conjugate vectors, it is not illogical to speculate that a viral integration system could also be incorporated into the design of the present system.

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References

1. Anderson, W.F. Prospects for human gene therapy. *Science* **226**, 401–409 (1984).
2. Anderson, W.F. Human gene therapy. *Science* **256**, 808–813 (1992).
3. Blaese, R.M., Mullen, C.A. & Ramsey, W.J. Strategies for gene therapy. *Path Biol* **41**(8), 672–676 (1993).
4. Friedmann, T. Progress toward human gene therapy. *Science* **244**, 1275–1281 (1989).
5. Helenius, A. Unpacking the incoming influenza virus. *Cell* **69**, 577–578 (1992).
6. Kielian, M.C. & Jungerwirth, S. Mechanisms of enveloped virus entry into cells. *Mol Biol Med* **7**, 17–31 (1990).
7. Choppin, P.W. & Scheid, A. The role of viral glycoproteins in adsorption, penetration, and pathogenicity of viruses. *Rev Infect Dis* **2**, 40–61 (1980).
8. Berkner, K.L. Development of adenovirus vectors for the expression of heterologous genes. *Biotechniques* **6**(7), 616–629 (1988).
9. Mastrangeli, A., Danel, C., Rosenfeld, M.A. *et al.* Diversity of airway epithelial cell targets for *in vivo* recombinant adenovirus-mediated gene transfer. *J Clin Invest* **91**(1), 225–234 (1993).
10. Stratford-Perricaudet, L.D., Makeh, I., Perricaudet, M. & Briand, P. Widespread long-term gene transfer to mouse skeletal muscles and heart. *J Clin Invest* **90**, 626–630 (1992).
11. McGrory, W.J., Bautista, D.S. & Graham, F.L. A simple technique for the rescue of early region 1 mutations into infectious human adenovirus type 5. *Virology* **163**, 614–617 (1988).
12. Karpati, G. & Acsadi, G. The potential for gene therapy in Duchenne muscular dystrophy and other genetic muscle diseases. *Muscle Nerve* **16**, 1141–1153 (1993).
13. Varmus, H.E. & Swanstrom, R. The molecular biology of tumor viruses: replication of retroviruses. In: *RNA Tumor Viruses*. Weiss, A., Teich, N., Varmus, H.E. & Coffin, J.M. (eds.), Cold Spring Harbor Laboratory: New York, 1984, pp. 369–512.
14. Eglitis, M.A. & Anderson, W.F. Retroviral vectors for introduction of genes into mammalian cells. *Biotechniques* **6**, 608–614 (1988).
15. Miller, D.G., Adam, M.A. & Miller, A.D. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol Cell Biol* **10**(8), 4239–4242 (1990).
16. Mannino, R.J. & Gould-Fogerite, S. Liposome mediated gene transfer. *Biotechniques* **6**, 682–690 (1988).
17. Loyter, A., Scangos, G.A. & Ruddle, F.H. Mechanism of DNA uptake by mammalian cells: fate of exogenously added DNA monitored by the use of fluorescent dyes. *Proc Natl Acad Sci USA* **79**, 422–426 (1982).
18. Wolff, J.A., Malone, R.W., Williams, P. *et al.* Direct gene transfer into mouse muscle *in vivo*. *Science* **247**, 1465–1468 (1990).
19. Wu, G.Y. & Wu, C.H. Receptor-mediated *in vitro* gene transformation by a soluble DNA carrier system. *J Biol Chem* **262**, 44299–4432 (1987).
20. Wu, G.Y., Wilson, J.M., Shalaby, F., Grossman, M., Shafritz, D.A. & Wu, C.H. Receptor-mediated gene delivery *in vivo*. Partial correction of genetic analbuminemia in nagase rats. *J Bio Chem* **266**, 14338–14342 (1991).
21. Wagner, E., Zenke, M., Cotten, M., Beug, H. & Birnstiel, M.L. Transferrin–polycation conjugates as carriers for DNA uptake into cells. *Proc Natl Acad Sci USA* **87**, 3410–3414 (1990).
22. Zenke, M., Steinlein, P., Wagner, E., Cotten, M., Beug, H. & Birnstiel, M.L. Receptor-mediated endocytosis of transferrin–polycation conjugates: an efficient way to introduce DNA into hematopoietic cells. *Proc Natl Acad Sci USA* **87**, 3655–3659 (1990).
23. Cotten, M., Langle-Rouault, F., Kirlappos, H. *et al.* Transferrin–polycation-mediated introduction of DNA into human leukemic cells: stimulation by agents that affect the survival of transfected DNA or modulate transferrin receptor levels. *Proc Natl Acad Sci USA* **87**, 4033–4037 (1990).
24. Ferkol, T., Kaetzel, C.S. & Davis, P.B. Gene transfer into respiratory epithelial cells by targeting the polymeric immunoglobulin receptor. *J Clin Invest* **92**(5), 2394–2400 (1993).
25. Wagner, E., Cotten, M., Foisner, R. & Birnstiel, M.L. Transferrin–polycation–DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. *Proc Natl Acad Sci USA* **88**, 4255–4259 (1991).
26. Zatloukal, K., Wagner, E., Cotten, M. *et al.* Transferrinfection: a highly efficient way to express gene constructs in eukaryotic cells. *Ann NY Acad Sci* **660**, 136–153 (1992).
27. Greber, U.F., Willetts, M., Webster, P. & Helenius, A. Stepwise dismantling of adenovirus 2 during entry into cells. *Cell* **75**(3), 477–486 (1993).
28. Pastan, I., Seth, P., FitzGerald, D. & Willingham, M. Adenovirus entry into cells: some new observations on an old problem. In: *Virus Attachment and Entry into Cells.*

Crowell, R.L. & Lonberg-Holm, K. (eds.) American Society for Microbiology: Washington, DC, 1986, pp. 141–146.
29. Defer, C. Belin, M.-T., Caillet-Boudin, M.-L. & Boulanger, P. Human adenovirus–host cell interactions: comparative study with members of subgroups B and C. *J Virol* **64**, 3661–3673 (1990).
30. Seth, P., FitzGerald, D., Ginsberg, H., Willingham, M. & Pastan, I. Evidence that the penton base of adenovirus is involved in potentiation of toxicity of *Pseudomonas* exotoxin conjugated to epidermal growth factor. *Mol Cell Biol* **4**, 1528–1533 (1984).
31. Fernandez-Puentes, C. & Carrasco, L. Viral infection permeabilizes mammalian cells to protein toxins. *Cell* **20**, 769–775 (1980).
32. FitzGerald, D.J.P., Padmanabhan, R., Pastan, I. & Willingham, M.C. Adenovirus-induced release of epidermal growth factor and pseudomonas toxin into the cytosol of KB cells during receptor-mediated endocytosis. *Cell* **32**, 607–617 (1983).
33. Curiel, D.T., Agarwal, S., Wagner, E. & Cotten, M. Adenovirus enhancement of transferrin–polylysine–mediated gene delivery. *Proc Natl Acad Sci USA* **88**, 8850–8854 (1991).
34. Philipson, L., Lonberg-Holm, K. & Pettersson, U. Virus–receptor interaction in an adenovirus system. *J Virol* **2**(10), 1064–1075 (1968).
35. Philipson, L. Structure and assembly of adenoviruses. *Curr Top Microbiol Immunol* **109**, 2–52 (1983).
36. Curiel, D.T., Wagner, E., Cotten, M. *et al.* High efficiency *in vitro* gene transfer mediated by adenovirus coupled to DNA–polylysine complexes via an antibody bridge. *Hum Gene Ther* **3**, 147–154 (1992).
37. Cotten, M., Wagner, E., Zatloukal, K., Phillips, S., Curiel, D.T. & Birnstiel, M.L. High-efficiency receptor-mediated delivery of small and large (48 kilobase) gene constructs using the endosome-disruption activity of defective or chemically inactivated adenovirus particles. *Proc Natl Acad Sci USA* **89**, 6094–6098 (1992).
38. Wagner, E., Zatloukal, K., Cotten, M. *et al.* Coupling of adenovirus to transferrin–polylysine–DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes. *Proc Natl Acad Sci USA* **89**, 6099–6103 (1992).
39. Michael, S.I., Huang, C.-H., Rømer, M.U., Wagner, E., Hu, P.-C. & Curiel, D.T. Binding-incompetent adenovirus facilitates molecular conjugate-mediated gene transfer by the receptor-mediated endocytosis pathway. *J Biol Chem* **268**(10), 6866–6869 (1993).
40. Curiel, D.T., Agarwal, S., Rømer, M.U. *et al.* Gene transfer to respiratory epithelial cells via the receptor-mediated endocytosis pathway. *Am J Respir Cell Mol Biol* **6**, 247–252 (1992).
41. Harris, C.E., Agarwal, S., Hu, P.-C., Wagner, E. & Curiel, D.T. Receptor-mediated gene transfer to airway epithelial cells in primary culture. *Am J Respir Cell Mol Biol* **9**, 441–447 (1993).
42. Cristiano, R.J., Smith, L.C. & Woo, S.L. Hepatic gene therapy: adenovirus enhancement of receptor-mediated gene delivery and expression in primary hepatocytes. *Proc Natl Acad Sci USA* **90**, 2122–2126 (1993).
43. Cristiano, R.J., Smith, L.C., Kay, M.A., Brinkley, B.R. & Woo, S.L. Hepatic gene therapy: efficient gene delivery and expression in primary hepatocytes utilizing a conjugated adenovirus–DNA complex. *Proc Natl Acad Sci USA* **90**, 11548–11552 (1993).
44. Ledley, F.D., Darlington, G.J., Hahn, T. & Woo, S.L. Retroviral gene transfer into primary hepatocytes: implications for genetic therapy of liver-specific functions. *Proc Natl Acad Sci USA* **84**(15), 5335–5339 (1987).
45. Gao, L., Wagner, E., Cotten, M. *et al.* Direct *in vivo* gene transfer to airway epithelium employing adenovirus–polylysine–DNA complexes. *Hum Gene Ther* **4**, 17–24 (1993).
46. Wilson, J.M., Grossman, M., Wu, C.H., Chowdhury, N.R., Wu, G.Y. & Chowdhury, J.R. Hepatocyte-directed gene transfer *in vivo* leads to transient improvement of hypercholesterolemia in low density lipoprotein receptor-deficient rabbits. *J Biol Chem* **267**(2), 963–967 (1992).
47. Chowdhury, N.R., Wu, C.H., Wu, G.Y., Yerneni, P.C., Bommineni, V.R. & Chowdhury, J.R. Fate of DNA targeted to the liver by asialoglycoprotein receptor-mediated endocytosis *in vivo*. *J Biol Chem* **268**(15), 11265–11271 (1993).
48. Findeis, M.A., Merwin, J.R., Spitalny, G.L. & Chiou, H.C. Targeted delivery of DNA for gene therapy via receptors. *Tibtech* **11**, 202–205 (1993).
49. Smith, T.A., Mehaffey, M.G., Kayda, D.B. *et al.* Adenovirus mediated expression of therapeutic plasma levels of human factor IX in mice. *Nat Genet* **5**, 397–402 (1993).